

A Pathobiont of the Microbiota Balances Host Colonization and Intestinal Inflammation

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SUMMARY

The gastrointestinal tract harbors a diverse microbiota that has coevolved with mammalian hosts. Though most associations are symbiotic or commensal, some resident bacteria (termed pathobionts) have the potential to cause disease. Bacterial type VI secretion systems (T6SSs) are one mechanism for forging host-microbial interactions. Here we reveal a protective role for the T6SS of *Helicobacter hepaticus*, a Gram-negative bacterium of the intestinal microbiota. *H. hepaticus* mutants with a defective T6SS display increased numbers within intestinal epithelial cells (IECs) and during intestinal colonization. Remarkably, the T6SS directs an anti-inflammatory gene expression profile in IECs, and CD4⁺ T cells from mice colonized with T6SS mutants produce increased interleukin-17 in response to IECs presenting *H. hepaticus* antigens. Thus, the *H. hepaticus* T6SS limits colonization and intestinal inflammation, promoting a balanced relationship with the host. We propose that disruption of such balances contributes to human disorders such as inflammatory bowel disease and colon cancer.

INTRODUCTION

The mammalian gastrointestinal (GI) tract represents an ecological niche of extraordinary microbial complexity (Hooper and Gordon, 2001). Humans and other mammals offer residence to ~1000 microbial species, which form an interkingdom bionetwork known to provide nutritional, metabolic, and immunologic benefits to the host (Ley et al., 2006). Although the magnitude of this evolutionarily dynamic interaction is undeniably vast, surprisingly few molecules that mediate host-microbiota associations have been described. Microbial secretion systems are universal mechanisms dedicated to molecular interactions between bacterial and host cells and can range from simple transporters to large, membrane-spanning structures. Type III secretion systems (T3SSs), T4SSs, and T6SSs function as multi-subunit complexes that translocate effector substrates across the double membrane of Gram-negative bacteria and act as a “needle and syringe” to inject molecules directly into eukaryotic cells (Raskin et al., 2006). The temporal and spatial profile of secreted molecules can have profound effects on host

biology. Therefore, these secretion systems represent well-evolved microbial strategies capable of mediating molecular interactions between the microbiota and mammals.

T6SS genes are present in over 25% of all sequenced bacterial genomes and have been largely associated with bacterial pathogenesis. In recent seminal studies, Mekalanos and colleagues reported that *Vibrio cholerae* requires T6SS genes to cause cytotoxicity in macrophages (Pukatzki et al., 2006). A set of molecules were identified as the first T6SS substrates: hemolysin coregulated protein (Hcp) and three valine-glycine-repeat (VgrG) proteins. Further evidence supporting a role for T6SSs in bacterial virulence has been demonstrated in other Gram-negative pathogens including *Pseudomonas aeruginosa* (Mougous et al., 2006), enteroaggregative *E. coli* (Dudley et al., 2006), and *Aeromonas hydrophila* (Suarez et al., 2008). However, the function of bacterial T6SSs is not limited to eliciting disease. A growing body of research has shown that T6SSs may modulate virulence (Bladergroen et al., 2003; Das et al., 2002; Parsons and Heffron, 2005). Therefore, in some contexts T6SSs may modulate bacterial virulence in order to “balance” the relationship between microbe and host.

T6SSs are almost exclusively found in α -, β -, and γ -proteobacteria (Bingle et al., 2008). *Helicobacter hepaticus* is unique in that it appears to contain the only bacterial genome in the epsilon (ϵ) subgroup of proteobacteria that encodes for a T6SS. *H. hepaticus*, a spiral microaerophilic bacterium, promotes inflammation in animal models of colon cancer and experimental colitis (inflammation of the colon). Interestingly, *H. hepaticus* only causes disease in immunocompromised animals that lack immune regulation and mount inflammatory responses toward intestinal bacteria (Erdman et al., 2009; Kullberg et al., 2001). Pathologies elicited by *H. hepaticus* remarkably resemble that of human disease based on molecular, cellular, and histological parameters. Furthermore, colonization with *H. hepaticus* causes a chronic inflammatory response similar to human inflammatory bowel disease (IBD), in contrast to infections with *Citrobacter rodentium* or *Salmonella typhimurium*, which induce acute and self-limiting infections. Therefore, the *H. hepaticus*-*Mus musculus* relationship represents a unique and invaluable tool in the study of intestinal pathologies highly similar to human disorders such as IBD and colon cancer.

Interestingly, multiple studies have shown that *H. hepaticus* sustains long-term colonization of the lower GI tract of wild-type mice without causing intestinal disease (reviewed in Solnick and Schauer, 2001). In fact, *H. hepaticus* colonization is highly endemic in most laboratory animal facilities but is largely unnoticed due to the absence of clinical disease. We have previously proposed that *H. hepaticus* acts as an intestinal pathobiont—i.e.,

a symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host (Mazmanian et al., 2008; Round and Mazmanian, 2009). The concept of pathobionts is supported by clinical data which reveal that in IBD patients with underlying genetic mutations, inflammation is targeted to specific members of the microbiota and not to infectious pathogens (Packey and Sartor, 2009). T cell responses and antibody reactivity during IBD target certain subsets of symbiotic microbes such as *Escherichia*, *Clostridium*, and *Enterococcus* species that are found in all people. Since *H. hepaticus* displays a potentially pathogenic association with its murine host similar to specific microbes found in humans, and the T6SS appears to mediate both symbiotic and pathogenic outcomes, we investigated a role for the *H. hepaticus* T6SS during intestinal inflammation.

Herein we report that the T6SS of *H. hepaticus* mediates critical protective functions during association with its mammalian host. In cell cultures, infection of intestinal epithelial cells (IECs) with *H. hepaticus* T6SS mutants results in increased bacterial association compared to wild-type bacteria. In animals, T6SS mutants colonize the lower GI tract to a higher degree. Most importantly, *H. hepaticus* defective in type VI secretion is unable to restrain potent innate and adaptive immune responses in an animal model of experimental colitis. Coculture experiments with IECs presenting *H. hepaticus* antigens result in higher levels of proinflammatory cytokines when incubated with T cells from T6SS mutant-colonized animals compared to wild-type-colonized animals. Thus, our findings reveal that *H. hepaticus* has evolved a T6SS as a mechanism to actively maintain a nonpathogenic, symbiotic relationship in the GI tract by regulating bacterial colonization and host inflammation. Disturbances in the dynamic molecular interaction between gut bacteria and the intestinal immune system therefore lead to exacerbated host inflammation. As intestinal bacteria profoundly influence host biology, our findings support an emerging hypothesis that alteration in the composition of the microbiota, known as dysbiosis, is a critical factor in various human inflammatory disorders such as IBD and colon cancer.

RESULTS

H. hepaticus Possesses a Canonical and Functional T6SS

The genome of *Helicobacter hepaticus* contains a 71 kb genomic island (termed HHG11 for *Helicobacter hepaticus* genomic island 1), which includes genes homologous to virulence factors (Suerbaum et al., 2003). Isolates missing the pathogenicity island are highly attenuated for inducing disease, and deletion of a 19 gene segment of HHG11 reduces virulence (Ge et al., 2008). However, the functions of the gene products encoded within HHG11 remain unclear. In recent years, studies have identified a protein secretion system referred to as a type VI secretion system (T6SS) (Raskin et al., 2006). We report herein that HHG11 encodes for a set of T6SS genes that are homologous to those found in *V. cholerae*, *P. aeruginosa*, and other Gram-negative microorganisms (see Table S1 available online). *H. hepaticus* contains 12 homologous T6SS genes that are clustered and arranged in a genomic organization similar to other T6SSs (Figure 1A). A homolog of the *icmF* gene, thought to encode for a structural

transmembrane protein of virtually all identified T6SSs, lies within this genomic locus (*H. hepaticus* gene HH0252). Furthermore, HHG11 encodes for homologs of Hcp (HH0243) and three VgrG proteins (HH0237, HH0242, HH0291), highly conserved translocated substrates that play key roles in T6SSs.

We constructed insertional mutants in the *icmF* or *hcp* genes, as previous studies in several pathogens have shown that deletion of these genes leads to T6SS defects. An antibiotic selection marker (erythromycin acetyl transferase, *eryR*) (Mehta et al., 2007) was inserted within each open reading frame by homologous recombination. PCR amplification verified integration of the *eryR* gene to create mutants Δ icmF and Δ Hcp (Figure 1B). To validate a functional defect in the T6SS, in vitro cultured bacteria were assayed by immunoblot for Hcp. In wild-type bacteria, Hcp was detected in the culture supernatant and cell pellet fraction as expected (Figure 1C). However, in the Δ icmF mutant, Hcp was absent from supernatant fractions and accumulated to a higher degree in the cell pellet, indicating that Hcp is still produced but not secreted in the absence of a functional T6SS. Therefore, deletion of the *icmF* homolog results in a secretion defect for Hcp, demonstrating functional inactivation of the T6SS.

Bacteria possessing T6SSs bind host cells into which substrates can be injected. Since *H. hepaticus* colonizes the murine gut and is in close association with intestinal crypts (see data below), we investigated the association of *H. hepaticus* with murine IECs. *H. hepaticus* was cocultured with the mouse IEC line MODE-K (Vidal et al., 1993) and examined for translocation of VgrG by confocal immunofluorescence microscopy. Labeling of MODE-K cells with antisera specific for the T6SS substrate VgrG showed diffuse staining upon incubation with wild-type bacteria (Figures 1D and 1E). Conversely, VgrG labeling of MODE-K cells incubated with Δ icmF mutant showed punctate staining patterns suggesting VgrG produced by T6SS mutants is bacterially associated. As whole bacterial antisera raised against *H. hepaticus* reveals a punctate staining pattern as well (Figure 1D), the diffuse staining of VgrG in wild-type bacteria suggests substrate translocation in the presence of MODE-K cells (Figure 1E). Taken together, our results demonstrate that deletion of T6SS genes results in mutants unable to secrete effector substrates during bacterial growth and coculture with IECs.

H. hepaticus Is Internalized into Murine IECs

Many enteric pathogens such as *Listeria monocytogenes* and *Shigella flexneri* have been shown to enter IECs during infection. In several cases, T3SSs either facilitate entry or are required for internalization (Galan and Wolf-Watz, 2006). To determine if *H. hepaticus* enters IECs, we infected bacteria with MODE-K cells and observed that both wild-type and T6SS mutants were capable of entering host cells (Figure 2A). Confocal microscopy demonstrated that all strains were found associated with the cell surface as well. Z stacks reconstructed from confocal images further revealed significant numbers of intact bacteria within the intracellular compartment of IECs (Movie S1). To determine if uptake was an active process, we incubated wild-type and T6SS mutants with MODE-K cells for 30 min in the presence or absence of cytochalasin D, an inhibitor of actin polymerization. Cells were subsequently treated with gentamicin to kill

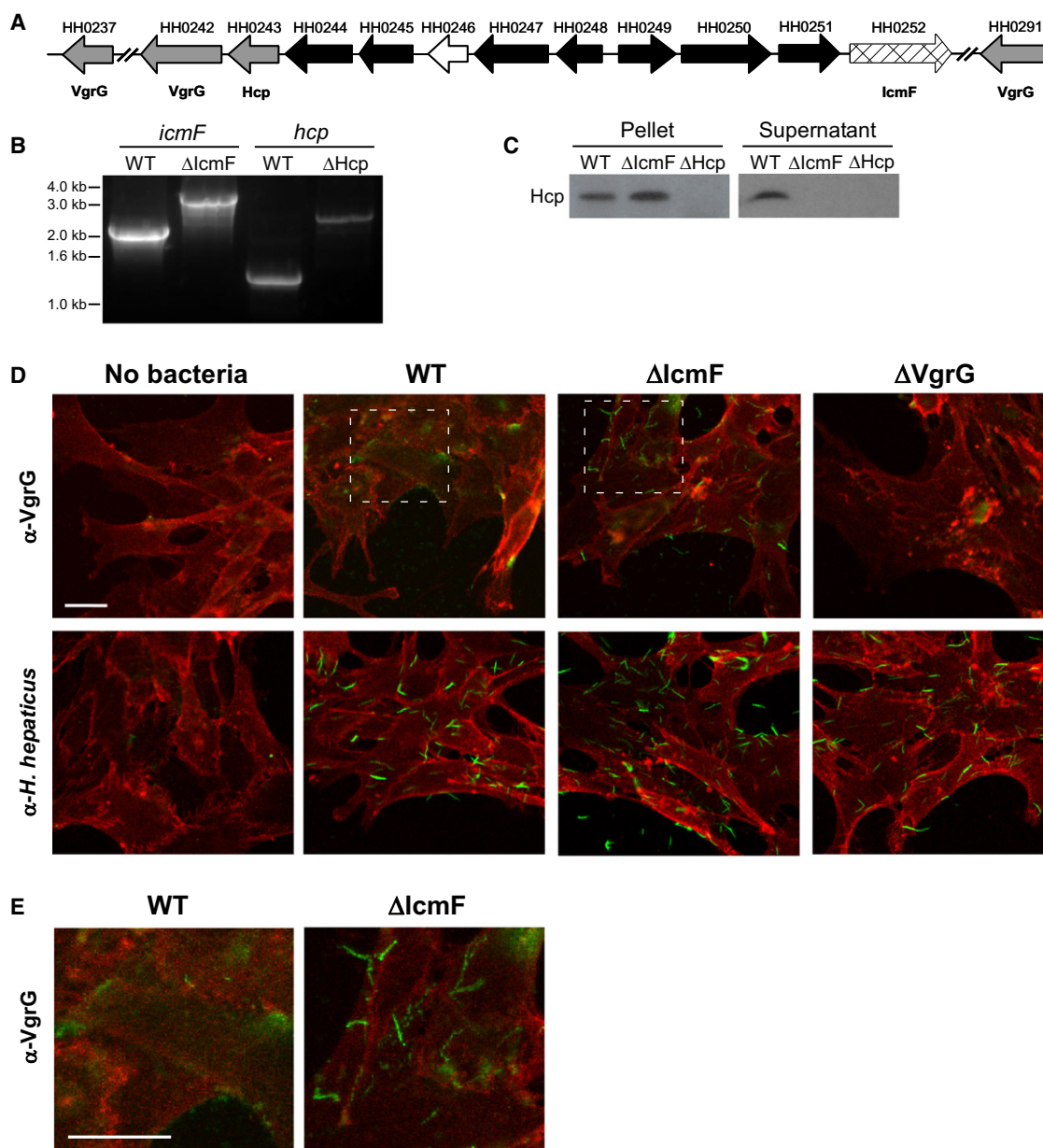


Figure 1. *H. hepaticus* Encodes for a Functional T6SS

(A) Schematic diagram of the genetic organization of *H. hepaticus* T6SS genes. Gray arrows represent *Hcp* and *VgrG* genes, cross-hatched arrow indicates *IcmF* homolog, black arrows represent T6SS homologs of unknown function, and white arrow indicates a gene nonhomologous to other T6SS genes. See also Table S1.

(B) Genomic DNA collected from mid-log cultures of WT, Δ *IcmF*, or Δ *Hcp* *H. hepaticus* was amplified by PCR using *icmF*- or *hcp*-specific primers. Insertion of the *eryR* gene was detected by a 1.1 kb increase in the resulting PCR band.

(C) Hcp is undetected in supernatants from Δ *IcmF* and Δ *Hcp* T6SS mutants. Equal amounts of mid-log bacterial cultures of WT, Δ *IcmF*, or Δ *Hcp* were centrifuged to separate bacterial pellets and supernatant. Supernatants were subsequently filtered to ensure removal of all bacteria. Bacterial pellets and supernatants were analyzed by western blot. Membranes were blotted with anti-Hcp antibody.

(D and E) Confocal images of bacteria incubated with MODE-K cells. WT, Δ *IcmF*, or Δ *VgrG* (HH0242) *H. hepaticus* were incubated with MODE-K cells for 5 hr. MODE-K cells were subsequently rinsed with PBS, fixed in 4% PFA, and stained for the eukaryotic cell membrane marker wheat germ agglutinin (red) and either *H. hepaticus* or VgrG (green). Outlined regions for WT and Δ *IcmF* in (D) are shown at higher magnification (E). Scale bar represents 20 μ m.

extracellular bacteria and plated for colony-forming units (CFUs). Figure 2B reveals that while all strains could be recovered from the intracellular compartment of MODE-K cells, cytochalasin D

inhibited bacterial uptake. Therefore, *H. hepaticus* appears to be actively internalized into cultured IECs through a requirement for actin rearrangement.

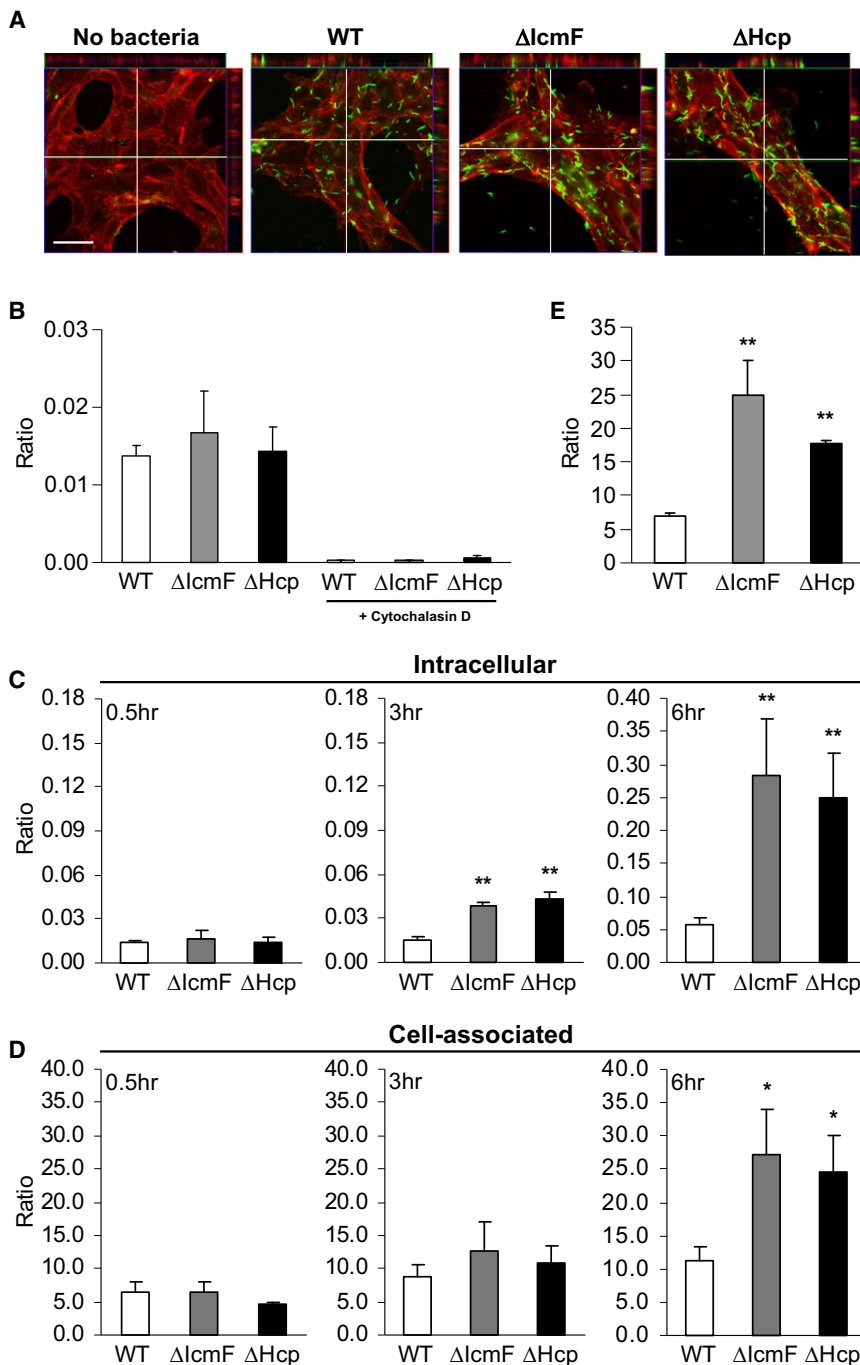


Figure 2. T6SS Mutants Display Higher Intracellular and Cell-Associated Accumulation in MODE-K Cells

(A) Confocal image of bacteria inside MODE-K cells. WT, Δ lcmF, or Δ Hcp was incubated with MODE-K for 6 hr. MODE-K cells were rinsed with PBS, fixed in 4% PFA, and stained for *H. hepaticus* (green) and the eukaryotic cell membrane marker wheat germ agglutinin (red). Scale bar represents 30 μ m. See also Movie S1.

(B) Cytochalasin D inhibits uptake of *H. hepaticus*. Prior to incubation with bacteria, MODE-K cells were treated with 10 μ M cytochalasin D for 1 hr. Bacteria were added at an moi of 100. After 0.5 hr incubation at 37°C under microaerophilic conditions, cells were treated with 100 μ g/ml gentamicin and intracellular bacteria plated for enumeration. Results are expressed as CFUs of intracellular bacteria divided by number of MODE-K cells. Error bars indicate SEM from three experiments.

(C and D) Gentamicin protection assay in which MODE-K cells were incubated with bacteria at an moi of 100. After 0.5, 3, or 6 hr incubation, media was replaced with 100 μ g/ml gentamicin for enumeration of intracellular bacteria (C) or without gentamicin for cell-associated bacteria (D). Cells were washed and bacteria plated for quantification. Ratios are expressed as CFUs of bacteria divided by number of MODE-K cells. Error bars indicate SEM from three to five experiments. * $p < 0.05$, ** $p < 0.01$ versus WT.

(E) Increased adherence of T6SS mutants is not dependent on bacterial internalization. Prior to coculture, MODE-K cells were treated with 10 μ M cytochalasin D. Bacteria were added at an moi of 100 for 6 hr at 37°C under microaerophilic conditions. Bacteria were plated for enumeration. Results are expressed as CFUs of bacteria divided by number of MODE-K cells. Error bars indicate SD from two experiments. ** $p < 0.01$ versus WT. See also Figure S1.

T6SS Mutants Display Increased Cell Association within IECs

We sought to determine if the T6SS affects bacterial internalization. MODE-K cells were incubated with *H. hepaticus*, treated with (or without) gentamicin, and plated for bacterial enumeration. At early time points (30 min), no differences were observed in the proportions of intracellular and cell-associated bacteria between wild-type and T6SS mutants (Figures 2C and 2D). Remarkably, by 3 and 6 hr, Δ lcmF and Δ Hcp had significantly higher levels of intracellular bacteria (Figure 2C). In addition,

these results reveal that the T6SS of *H. hepaticus* limits intracellular bacterial numbers within IECs.

To investigate if the increased cell association by T6SS mutants was due to increased adherence, we blocked internalization of *H. hepaticus* into MODE-K cells. Cytochalasin D was added for the duration of the coculture (6 hr). In the absence of bacterial internalization, T6SS mutants still exhibited an increase in adherence to the extracellular surface of MODE-K cells (Figure 2E). An increase in cell adherence in the absence of internalization was also observed for T6SS mutants when MODE-K

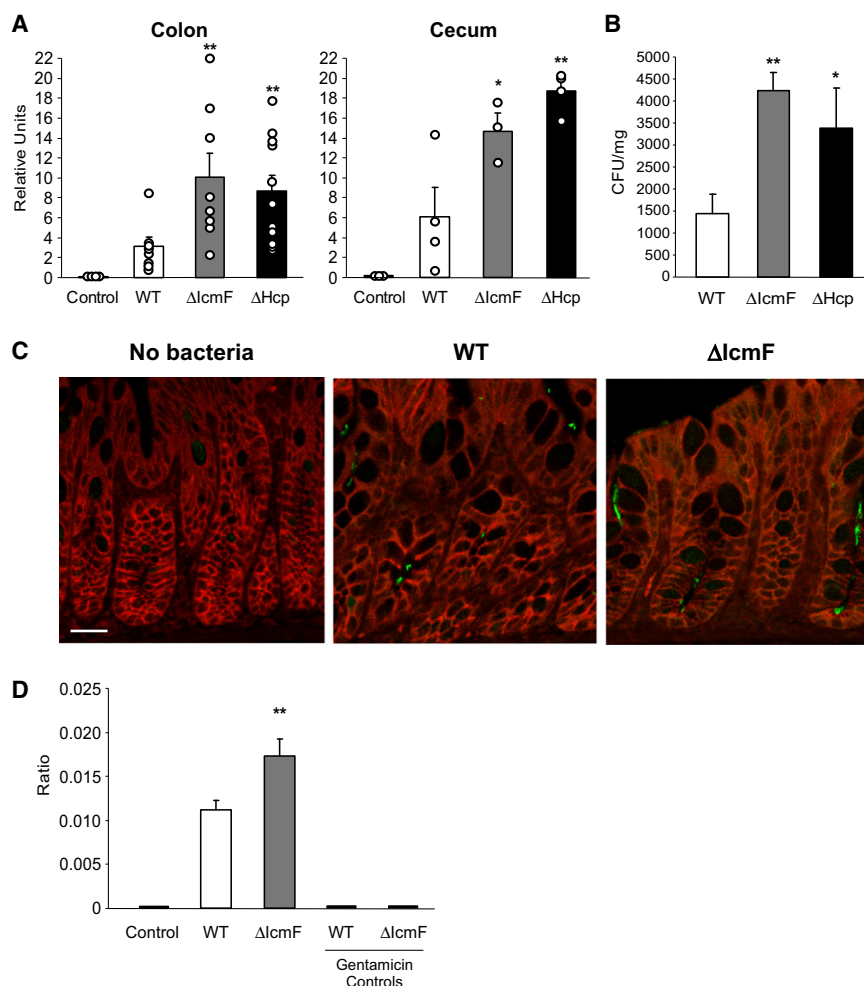


Figure 3. T6SS Mutants Have Increased Colonization Levels within the Colon

(A) ΔlcmF - and ΔHcp -monocolonized animals have greater amounts of *H. hepaticus* 16S rRNA in the colon and cecum compared to WT-monocolonized animals. RNA was collected from colon and cecum. Levels of 16S were quantified by qRT-PCR using *H. hepaticus*-specific 16S primers. Error bars show SEM, $n = 4$ –11 animals per group. * $p < 0.05$, ** $p < 0.01$ versus WT. See also Figure S2A.

(B) ΔlcmF - and ΔHcp -monocolonized animals have higher levels of viable *H. hepaticus* in the colon compared to WT-monocolonized animals. Colon tissues were homogenized in BHI and plated for quantification. Bacterial numbers were normalized to colon tissue weights. Units are expressed as CFUs per mg of tissue. Error bars show SEM from $n = 4$ animals per group. * $p < 0.05$, ** $p < 0.01$ versus WT.

(C) *H. hepaticus* is found in colonic intestinal crypts. Colons from WT- and ΔlcmF -monocolonized mice were paraffin embedded and sectioned. Colon sections were stained for *H. hepaticus* (green) and E-cadherin (red). Animals were colonized for 8 weeks. Scale bar represents 20 μm . See also Figures S2B–S2D.

(D) ΔlcmF -colonized animals have increased levels of intracellular *H. hepaticus* compared to WT-colonized animals. Purified IECs were treated with gentamicin either prior to or following lysis with saponin (which selectively permeabilizes eukaryotic membranes). Bacteria were enumerated by plating on TVP (trimethoprim, vancomycin, polymyxin B) plates which are known to select for *H. hepaticus*, as confirmed by control mice which received no *H. hepaticus*. In “Gentamicin Controls,” IECs were lysed and then treated with gentamicin. Ratio is expressed as CFUs of bacteria divided by number of IECs. Error bars indicate SEM from $n = 12$ animals per group. ** $p < 0.01$ versus WT. See also Figure S2E.

cells were fixed with 4% paraformaldehyde prior to coincubation (data not shown). Despite the increase in MODE-K adherence, the T6SS mutants did not affect the viability of MODE-K cells compared to wild-type bacteria. Staining for MODE-K cell apoptosis with Annexin V and propidium iodide showed that neither wild-type nor T6SS mutants had a significant effect on IEC viability (Figure S1B). Our studies are consistent with previous reports (for other bacteria) suggesting that T6SSs limit entry of additional microorganisms once bacteria have already gained access to the host cytoplasm (Ma et al., 2009).

T6SS Mutants Display Increased Colonization of the Murine Intestine

Germ-free animals (devoid of all microorganisms from birth) were employed to measure in vivo intestinal colonization. Mice were monocolonized with wild-type, ΔlcmF , or ΔHcp *H. hepaticus* (ranging from 2 to 13 weeks of colonization), at which time RNA was isolated from colonic tissue. Colonization levels were determined by quantitative RT-PCR (qRT-PCR) for *H. hepaticus*-specific 16S ribosomal RNA, as previously described (Nanda Kumar et al., 2008). Interestingly, RNA extracted from

the ceca and colons of monocolonized animals revealed that *H. hepaticus* colonization by ΔlcmF and ΔHcp mutants was increased compared to wild-type (Figure 3A). In vitro analysis of RNA (and DNA) levels demonstrated that mutating the T6SS does not affect 16S rRNA expression (Figure S2A). Accordingly, plating for viable bacterial CFUs from homogenized colonic tissue confirmed significantly elevated levels of colonization for T6SS mutants (Figure 3B). We observed a similar increase for T6SS mutants following colonization for up to 3 months in two strains of germ-free mice, outbred Swiss Webster and inbred C57Bl/6 mice (data not shown). Images of colon sections from monocolonized animals showed that both wild-type and ΔlcmF were found intimately associated with host cells (Figure 3C). Consistent with previous reports for wild-type *H. hepaticus* (Kullberg et al., 2002), we did not observe any cases of intestinal disease in colonized animals. Examination of colonic tissues (e.g., epithelial hyperplasia, infiltration of leukocytes, crypt abscesses) revealed no signs of histopathology (Figure S2B and data not shown). qRT-PCR analysis of colonic tissues for proinflammatory cytokines showed comparable levels of tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) between

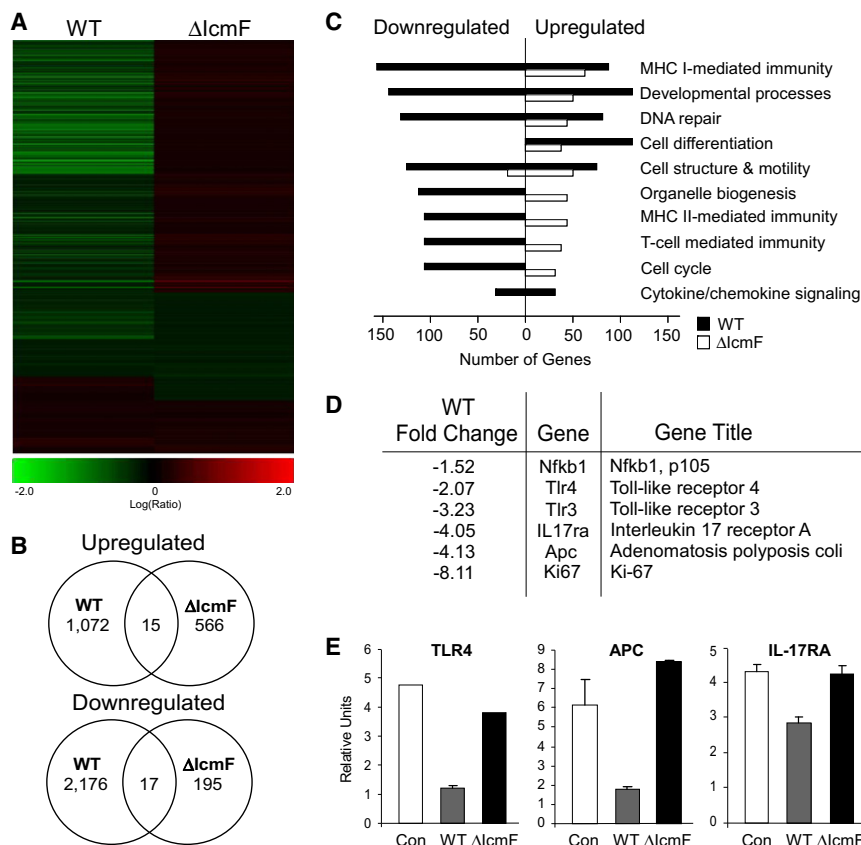


Figure 4. Wild-type *H. hepaticus* Induces a Wide Repertoire of Responses in MODE-K Cells

RNA was harvested from MODE-K cells incubated for 6 hr with either wild-type *H. hepaticus*, Δ lcmF mutant, or no bacteria and analyzed by an Agilent Whole Mouse Genome Microarray. Gene expression of MODE-K cells incubated with bacteria was compared to transcript levels from RNA of untreated MODE-K cells. Only genes with a p value <0.5 and fold change >1.5 were used for subsequent analysis.

(A) Heat-map analysis of MODE-K gene expression in the presence of WT or Δ lcmF shows massive downregulation by wild-type *H. hepaticus*.

(B) Venn diagram showing up- and down regulation of gene expression in the presence of WT or Δ lcmF.

(C) Gene ontology analysis of changes in MODE-K transcript levels for various functional groups. Wild-type bacteria downregulate numerous cellular pathways.

(D) Fold change of select innate and adaptive immune genes in the presence of WT *H. hepaticus*.

(E) qRT-PCR analysis of RNA from MODE-K cells for genes associated with inflammation and colon cancer. Error bars show SEM.

germ-free and monocolonized animals (Figure S2C). In addition, the percentage of CD4⁺TCR β ⁺ cells in the mesenteric lymph nodes (MLNs) did not differ between groups (Figure S2D).

To investigate the intracellular association of *H. hepaticus* within IECs in vivo, we colonized SPF (specific pathogen-free) C57Bl/6 mice with wild-type, Δ lcmF, or no *H. hepaticus*. Colonic IECs were purified and treated with gentamicin either prior to or following IEC lysis. Internalized bacteria were enumerated as the gentamicin-resistant population. Interestingly, Δ lcmF showed increased intracellular numbers in IECs compared to wild-type bacteria (Figure 3D). This increase in bacterial internalization by Δ lcmF was also observed in IECs recovered from the colons and ceca of C57Bl/6 *Rag1*^{-/-} and *IL-10*^{-/-} mice (Figure S2E). IECs lysed with saponin prior to incubation with gentamicin showed no viable *H. hepaticus* bacteria, demonstrating the complete killing of bacteria (Figure 3D). Our findings reveal that, contrary to most secretion systems of enteric bacteria which promote infections, the *H. hepaticus* T6SS limits bacterial numbers during colonization of the mouse intestine.

The T6SS Directs an Anti-Inflammatory Gene Expression Profile in IECs

Based on the evidence from these studies of T6SS mutants, it appears that *H. hepaticus* intimately interacts with IECs. To appreciate the magnitude of this interaction, we examined the gene expression profile of MODE-K cells incubated with either wild-type or Δ lcmF bacteria. GeneChip analysis showed clear downregulation of numerous transcripts in MODE-K cells cocul-

tured with wild-type bacteria possessing a functional T6SS (Figures 4A and 4B). Examination of various gene families exhibited an unmistakable pattern of transcriptional repression by wild-type *H. hepaticus*, including many immune-related genes (Figure 4C). Previous studies have shown that *H. hepaticus* reduces activation of innate immune molecules such as Toll-like receptor 4 (TLR4) in IECs (Sterzenbach et al., 2007). We reveal by both microarray and qRT-PCR analysis of MODE-K RNA that suppression of TLR4 expression by *H. hepaticus* is T6SS mediated (Figures 4D and 4E). Moreover, wild-type bacteria reduced the transcript levels for several additional mediators of innate (NF- κ B, TLR3) and adaptive immunity (interleukin-17 receptor, IL-17RA). *H. hepaticus* promotes colon cancer in animal models; the molecular mechanisms underlying this outcome are unknown. Intriguingly, our data show that T6SS may serve a protective activity in the development of colorectal cancer, as a critical oncogene in hereditary colon cancers (Adenomatosis polyposis coli, APC) and a marker for cell proliferation during epithelial hyperplasia (Ki-67) are significantly reduced by wild-type bacteria (Figures 4D and 4E). The extent of the contribution by T6SS to *H. hepaticus*-mediated cancer remains to be determined. Collectively, these studies strongly support a model whereby the T6SS of *H. hepaticus* functions to reduce immune responses elicited by IECs during bacterial colonization.

H. hepaticus T6SS Mutant Elicits Elevated Intestinal Inflammatory Responses

During homeostasis, a defined balance exists among commensal, symbiotic, and pathobiotic bacteria in the intestinal microbiota. We and others have hypothesized that disturbances in this equilibrium may lead to intestinal disorders in humans

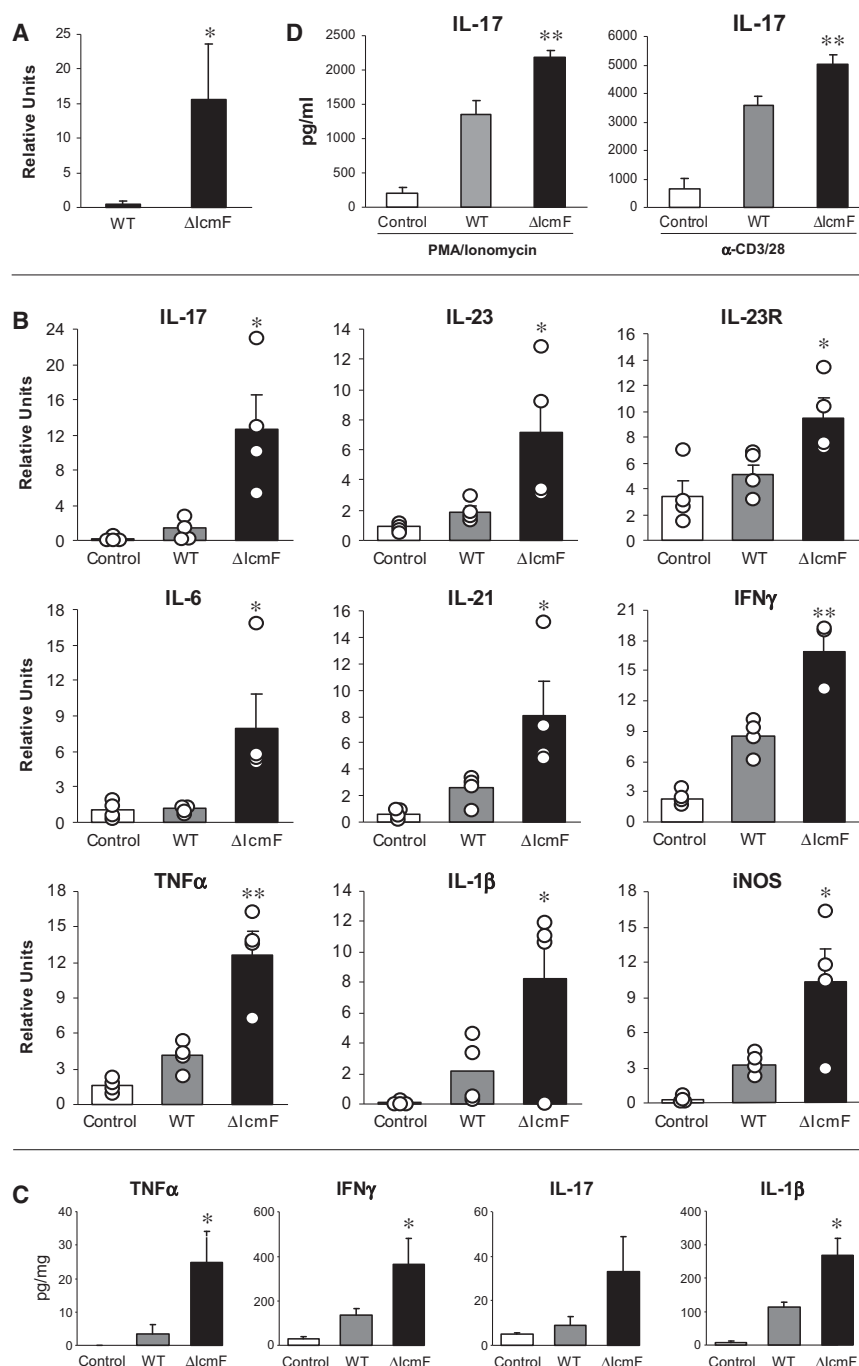


Figure 5. T6SS Mutant Leads to Greater Inflammation in the Colon in the Rag Adoptive Transfer Model of Colitis

(A) Δ lcmF-colonized animals have greater amounts of *H. hepaticus* 16S rRNA in the colon compared to WT-colonized animals. RNA was collected from colons. Levels of 16S were quantified by qRT-PCR. Data are representative of three experiments, with $n = 4$ per group. Error bars show SEM. * $p < 0.05$ versus WT.

(B) RNA from the colon was analyzed by qRT-PCR for inflammatory cytokines. Experimental values were normalized against L32. Results are representative of five experiments, with $n = 4$ per group. Each circle represents an individual animal. Error bars show SEM. * $p < 0.05$, ** $p < 0.01$ versus WT. See also Figure S3.

(C) Colons were cultured ex vivo for 24 hr. Supernatants were assayed for cytokine by ELISA. Samples were normalized to total protein. Data are representative of two experiments, with $n = 4$ per group. Each circle represents an individual animal. Error bars show SEM. * $p < 0.05$ versus WT.

(D) Mesenteric lymph nodes pooled from each experimental group were restimulated with either PMA and ionomycin, or α -CD3 and α -CD28 for 24 hr. Supernatants were assayed by ELISA. Results are from two experiments, with $n = 4$ mice per group. Error bars show SEM. ** $p < 0.01$ versus WT.

with naive, pathogenic CD4⁺CD45Rb^{high} T cells to provide immune cells that can react to the microbiota. In the absence of *H. hepaticus*, SPF T cell recipient animals remain healthy. However, in the presence of *H. hepaticus*, mice develop a profound inflammatory response in the colon (Mazmanian et al., 2008). To test the effects of T6SS, we colonized T cell-reconstituted *Rag1*^{-/-} mice with wild-type or Δ lcmF strains of *H. hepaticus*. Initially, we examined colonization levels and again observed a significant increase by the T6SS mutant (Figure 5A). Most importantly, colonization with Δ lcmF led to higher levels of intestinal inflammation compared to wild-type (Figure 5B). qRT-PCR of RNA from colons revealed that animals colonized with the T6SS mutant displayed considerably increased cyto-

kine transcript levels of numerous innate and adaptive immune responses. Many cytokines associated with the inflammatory T-helper 17 (T_H17) arm of immunity were increased in response to Δ lcmF—IL-17, IL-21, IL-23, and IL-23R—suggesting the T6SS may help in controlling exaggerated T_H17 responses toward bacteria. The proinflammatory cytokine TNF- α and inducible nitric oxide synthase (iNOS) were also significantly increased in response to the T6SS mutant. TNF- α and iNOS are highly expressed during *H. hepaticus*-induced colon inflammation and carcinogenesis and are believed to be strong contributors to

(reviewed in Packey and Sartor, 2009; Round and Mazmanian, 2009). Dysbiosis can result from an overgrowth of pathobionts or a loss of beneficial commensal bacteria, leading to elevated host inflammation toward the microbiota. We therefore tested this hypothesis through association of animals with T6SS mutants to induce dysbiosis by experimentally increasing colonization of a defined pathobiont.

The T cell transfer model of experimental colitis highlights the intricate interaction between mice and *H. hepaticus*. In this model, T and B cell-deficient *Rag1*^{-/-} mice are reconstituted

disease (Erdman et al., 2009). Interestingly, there was no marked difference in intestinal pathology by measures of cell proliferation, cellular infiltrates, and abscess formation between wild-type and Δ lcmF mutant-colonized animals (Figure S3A). Histopathology analysis by a blinded pathologist verified similar colitis scores between wild-type and mutant-colonized animals (Figure S3B). Although more subtle phenotypes cannot be excluded, the lack of increased disease by T6SS is not surprising given the fact that wild-type bacteria elicit very pronounced disease in the T cell transfer model. To measure inflammatory protein levels, organ cultures in which unstimulated colon sections are cultured ex vivo, and supernatants assayed by ELISA showed an increase in the inflammatory molecules TNF- α , IL-1 β , and IL-17 in tissues harvested from Δ lcmF mutant-colonized animals compared to wild-type (Figure 5C). Furthermore, MLN cells from Δ lcmF-colonized animals that were restimulated in vitro with PMA/ionomycin or T cell stimuli (α -CD3/ α -CD28) released increased IL-17 during in vitro cultures (Figure 5D). Analysis of the ceca also showed similar patterns of increased T6SS mutant colonization and elevated proinflammatory cytokine responses (Figures S3C–S3E). Collectively, these results reveal that experimentally induced dysbiosis results in increased inflammatory responses from both the innate and adaptive immune systems, and the T6SS of *H. hepaticus* functions to reduce intestinal inflammation during colonization.

IECs Are Capable of Stimulating CD4⁺ T Cell Responses with *H. hepaticus* Antigen

In the GI tract, IECs form a monolayer barrier that separates luminal contents from underlying host cells. Though IECs contribute to innate immunity by sensing through TLRs and secreting antimicrobial peptides, IECs are generally not believed to mediate adaptive immunity, unlike professional antigen-presenting cells (APCs) such as dendritic cells and macrophages. Interestingly, studies have shown that IECs are capable of expressing major histocompatibility complex (MHC) class II proteins (Bland and Warren, 1986; Mayer et al., 1991), and IEC presentation of antigens to CD4⁺ T cells via MHC class II molecules can result in lymphocyte proliferation (Westendorf et al., 2009; Vidal et al., 1993). MODE-K cells increase MHC class II expression in response to IFN γ treatment, when stained with antibodies directed against the I-A^k haplotype (Figure 6A) or to a nonpolymorphic region of the I-A molecule (Figure S4). The T cell activating costimulatory molecules CD80 (B7.1) and CD86 (B7.2) were also increased (data not shown). To determine if IECs could present *H. hepaticus* antigens to T cells, MODE-K cells treated for 7 days with IFN γ were pulsed with soluble *Helicobacter* antigens (SHeAg). After 24 hr, MODE-K cells were washed and cocultured with CD4⁺ T cells from MLNs of SPF *Helicobacter*-free C3H/HeJ animals. Total RNA was analyzed by qRT-PCR for cytokine levels. Cocultures that had been treated with SHeAg showed an increase in numerous proinflammatory cytokine transcripts, such as IL-17, IFN γ , and TNF- α (Figure 6B). These responses are T cell specific, as no cytokine production was observed in the absence of T cells.

Antigen-Specific CD4⁺ T Cell Responses Are Increased in Animals Colonized with *H. hepaticus* T6SS Mutants

We investigated the hypothesis that IEC presentation of *H. hepaticus* antigens elicits increased proinflammatory responses

from CD4⁺ T cells from animals colonized with T6SS mutants. MODE-K cells were pulsed with SHeAg or left untreated, washed, and incubated with purified CD4⁺ T cells from mice colonized with either wild-type, Δ lcmF, Δ Hcp, or no *H. hepaticus*. In the presence of SHeAg, T cells from uncolonized and wild-type *H. hepaticus*-colonized animals produced comparable levels of IL-17 (Figure 6C). This illustrates that previous exposure to *H. hepaticus* antigens does not augment immune responses relative to naive animals, consistent with our findings that wild-type bacteria do not induce inflammation. In contrast, CD4⁺ T cells harvested from both T6SS mutant-colonized animals elicited significantly increased levels of IL-17 compared to cells from wild-type-colonized animals. This response was antigen specific, as no cytokine was produced in the absence of SHeAg. Therefore, colonization of animals with T6SS mutants leads to the generation of an increased Th17 cell response in the gut, revealing that *H. hepaticus* evolved this molecular mechanism to restrain unwanted intestinal inflammation. As Th17 cell responses are important mediators of IBD and colon cancer in experimental animals (Hue et al., 2006), our data suggest that increased gut inflammation in reaction to dysbiosis of the microbiota may be crucial in the onset and/or progression of human intestinal diseases.

DISCUSSION

Host-bacterial interactions (whether beneficial or harmful) are defined by a dynamic exchange of molecules that mediate various biological outcomes. We characterize herein that the T6SS of *H. hepaticus* limits colonization of animals and actively suppresses innate and adaptive immune responses. Although T6SSs have been largely studied in the context of bacterial virulence, growing evidence supports the notion that T6SSs may have also evolved for nonpathogenic purposes in symbiotic bacteria. Previous studies have shown that *H. hepaticus* can reduce activation of TLR4 and TLR5 (Sterzenbach et al., 2007). Intriguingly, Kullberg et al. demonstrated that anti-inflammatory regulatory T cells (Tregs) from *H. hepaticus*-infected animals are able to prevent intestinal inflammation when transferred to naive mice (Kullberg et al., 2002). As Treg cells are known to suppress Th1 and Th17 cell responses, these data correlate well with our findings. However, the molecular mechanism(s) underlying these observations has remained elusive. A large deletion of the HHG11 has been reported to reduce inflammation caused by *H. hepaticus* resulting in the absence of typhocolitis in *IL-10*^{-/-} mice (Ge et al., 2008). Though differences arising from animal models cannot be excluded, we speculate that additional virulence factors (not T6SS components) within HHG11 may elicit proinflammatory responses. In support of this notion, several HHG11 genes bear homology to known toxins (data not shown). Prospective studies will determine the precise function of gene products within HHG11 and their effects on the induction of pathology in animal hosts. Collectively, however, we reveal herein that type VI secretion functions to attenuate both innate and adaptive immunity to *H. hepaticus*. A summary of these findings is depicted in Figure 7, illustrating that the T6SS of *H. hepaticus* may shape an immunologically tolerant host immune system (i.e., reducing TLR, Th17 responses and promoting Tregs) through its interaction with IECs. Though

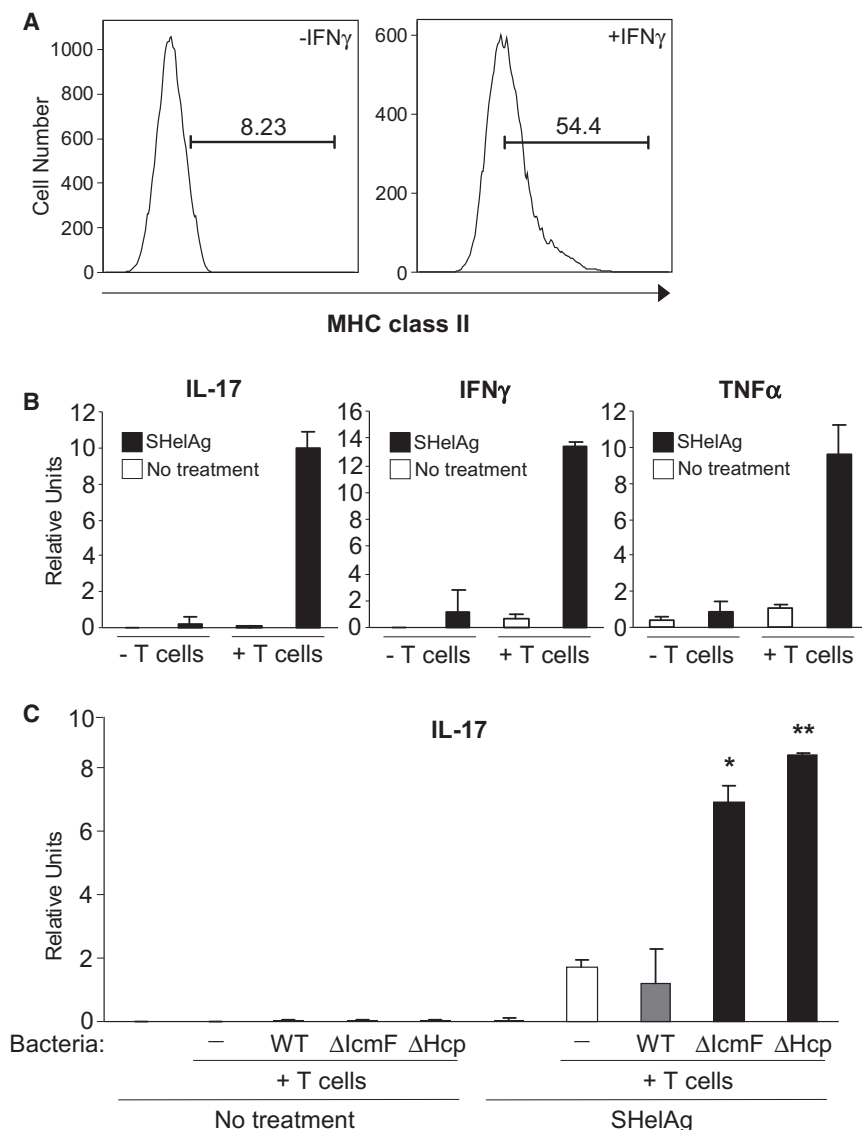


Figure 6. MODE-K Cells Are Capable of Stimulating CD4⁺ T Cell Responses with *H. hepaticus* Antigens

(A) MODE-K cells were treated with 100 U/ml of IFN γ for 7 days or left untreated. Cells were removed from the plate with trypsin, stained for MHC class II antigens, and analyzed by flow cytometry. See also Figure S4.

(B) MODE-K cells pretreated with 100 U/ml of IFN γ for 7 days were pulsed with either 20 μ g/ml of soluble *H. hepaticus* antigen (SHelAg) from wild-type bacteria or no SHelAg for 24 hr. Media was changed prior to addition of CD4⁺ T cells harvested from syngeneic C3H/HeJ *Helicobacter*-free animals or no T cells. The ratio of MODE-K:T cells was 1:10. Cells were cocultured for 3 days. RNA was collected from total cells and analyzed by qRT-PCR. Error bars indicate SD from two independent experiments.

(C) MODE-K cells pretreated with IFN γ were pulsed with either 20 μ g/ml of SHelAg from wild-type bacteria or no SHelAg for 24 hr. Media was changed prior to addition of CD4⁺ T cells collected from animals colonized with wild-type, Δ lcmF, Δ Hcp, or no *H. hepaticus*.

The ratio of MODE-K:CD4⁺ T cells was 1:10. Cells were cocultured for 3 days. RNA was collected from total cells and analyzed by qRT-PCR. Error bars indicate SD from two independent experiments. * $p < 0.05$, ** $p < 0.01$ versus WT treated with SHelAg.

intestinal human *Helicobacters* do not appear to be pathogens, as the majority of colonized people are asymptomatic. Unlike opportunistic pathogens that may not permanently colonize a host, understanding the dynamic molecular relationship between *Helicobacters* and mammals may provide paradigms for studies into human diseases caused by dysbiosis of probiotics.

numerous aspects of this host-bacterial interface remain to be determined, the T6SS appears to promote a symbiotic relationship between *H. hepaticus* and mammals.

Understanding how *H. hepaticus* is able to maintain a long-term, nonpathogenic symbiosis with its murine host will be critical for insight into the biology of human intestinal *Helicobacters*. A handful of studies have implicated at least two species with tropism for humans, *Helicobacter cinaedi* and *Helicobacter fennelliae*, as being associated with enterocolitis, diarrhea, and bacteremia in a portion of infected patients (Flores et al., 1990; Fox et al., 2000). Moreover, studies have shown a higher association of *Helicobacter* species in the intestinal tract of Crohn's disease and IBD patients compared to healthy patients, suggesting a potential role in pathogenicity (Bohr et al., 2004; Laharie et al., 2009). An important observation from these studies is that intestinal *Helicobacter* species are also detected in healthy patients. Similar to *Helicobacter pylori*, whereby only 1% of colonized people develop gastric ulcers or gastric cancer,

The complex consortium of microbes within our GI tract actively shapes mammalian immune responses (Mazmanian et al., 2005). The microbiota has been implicated in numerous human disorders such as IBD, colon cancer, allergies, asthma, and type 1 diabetes (Kinross et al., 2008; Mazmanian et al., 2008; Penders et al., 2007; Wen et al., 2008), highlighting the importance of understanding the individual species that make up a "healthy" microbiota. Numerous investigations have shown a significant alteration in the microbiota of patients with IBD (Frank et al., 2007; Scanlan et al., 2006). A recent metagenomic (culture-independent analysis) case-control study comparing the microbiota of patients with IBD to that of non-IBD controls revealed a statistically significant difference between the microbial compositions (Frank et al., 2007). Our understanding of how dysbiosis affects IBD is still preliminary. Further, genetic factors play an important role in the pathogenesis of IBD. Polymorphisms in bacterial-sensing (*NOD2/Card15*) (Hampe et al., 2001), autophagy (*ATG16L1*) (Hampe et al., 2007), and T cell

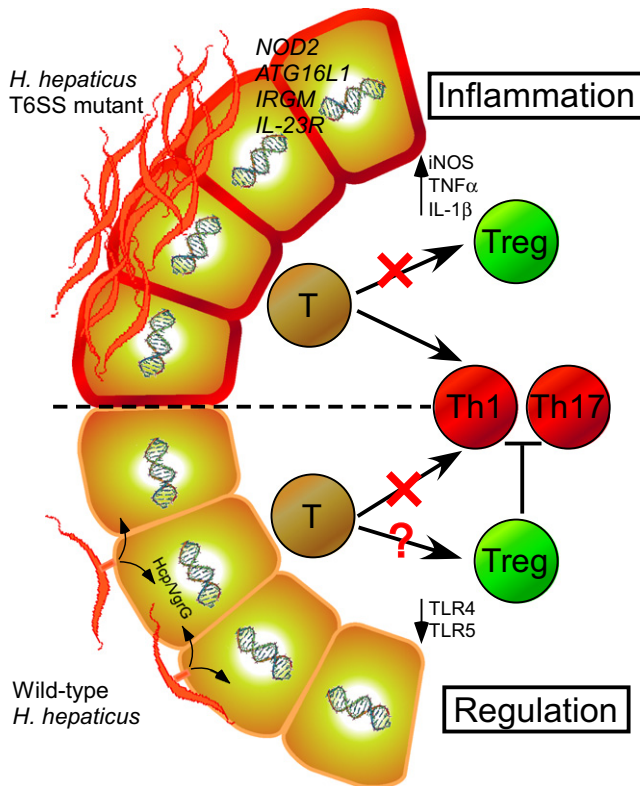


Figure 7. Proposed Interactions between *H. hepaticus* T6SS and the Intestinal Immune Response during Colonization

During prolonged intestinal colonization of animals, *H. hepaticus* intimately contacts the epithelium and uses its T6SS to create a tolerogenic immune environment (possibly through downregulating TLR expression and/or promoting Treg development). Crosstalk between host and bacteria maintains a balanced symbiotic interaction. This balance can be disturbed by genetic mutations associated with IBD (*NOD2*, *ATG16L1*, *IRGM*, *IL-23R*) and/or dysbiosis caused by external disturbances (e.g., antibiotics, enteric infections, diet, etc.), which may result in elevated immune responses (increased Th17) in genetically susceptible hosts. Based on our and previous studies, it appears that the combination of host genotype and microbial status contributes to intestinal disease.

immunity (*IL-23R*) (Duerr et al., 2006) genes have highlighted the connection between microbes and inflammation in IBD (Figure 7). However, genetic variations appear to predispose, but not predict, disease development as concordance rates between monozygotic twins are only 30% for IBD, and many people with polymorphisms for IBD-related genes are healthy. Thus, environmental factors play a significant role in disease. Mounting evidence predicts that IBD, at least in part, results from dysbiosis of the normal microbiota (O'Hara and Shanahan, 2006). The convergent contributions of the host genetic landscape and epigenetic variables (i.e., the microbiota) should therefore be considered in evaluating the cause of complex immunologic diseases in humans. Our findings predict that discrete and identifiable bacterial species of the microbiota can drive intestinal inflammation if their "balance" with the host is altered. From this perspective, therapeutics which selectively target pathobionts may prove invaluable as a treatment for intestinal diseases such as IBD and colon cancer.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Helicobacter hepaticus ATCC51449 (ATCC) was cultured on Brucella agar plates with 5% sheep's blood (Teknova) or in BHI with 10% FBS. Cultures were grown at 37°C in 1% O₂, 10% CO₂, and 10% H₂. For construction of mutants, ~2 kb fragments of *HH0252* (*IcmF*), *HH0243* (*Hcp*), and *HH0242* (*VgrG*) were PCR amplified and ligated into pGEMT (Promega). An erythromycin resistance gene digested from pSLB167 (Mehta et al., 2007) was inserted within the ORFs. Plasmid construction was carried out using *E. coli* JM109 with erythromycin and ampicillin used at 150 and 100 µg/ml, respectively. Plasmid was introduced into *H. hepaticus* by electroporation. Mutants were selected on plates with 5 µg/ml erythromycin.

MODE-K Cell Culture

MODE-K cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 mM HEPES at 37°C in 5% CO₂ incubator. Cells were passaged using trypsin-EDTA. For bacterial incubations, media were changed to UltraDOMA-PF media (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, nonessential amino acids, 1 mM sodium pyruvate, and 0.5 mM β-mercaptoethanol. Cytochalasin D (Sigma) was used at 10 µM and added 1 hr prior. For gentamicin protection assays, bacteria were added at an moi of 100. Incubations were carried at 37°C in 1% O₂. MODE-K cells were washed in PBS and media added with or without 100 µg/ml gentamicin for 2 hr at 37°C. MODE-K cells were rinsed with PBS, lysed with 0.1% saponin for 15 min at 25°C, and plated for bacterial quantification.

Generation of *H. hepaticus* Antibodies

Fragments (~900 bp) *HH0243* (*Hcp*) and *HH0242* (*VgrG*) were cloned into pQE30 6xHis-tagged expression vector (QIAGEN) and transformed into *E. coli* JM109. *E. coli* were grown at 25°C with 0.5 mM IPTG for 5 hr. Peptides were purified using Ni-NTA columns (QIAGEN) and injected into chickens for antibody production (QED Bioscience). Antibodies were collected from eggs using the EGGstract IgY kit (Promega).

Immunohistochemistry

MODE-K cells were fixed in 4% PFA. Paraffin-embedded tissues were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval in 10 mM sodium citrate (pH 6.0) was carried out for 20 min at 95°C. PBS with 5% FBS was used to block and dilute antibodies. Wheat germ agglutinin conjugated to tetramethylrhodamine was used at 1:1000 for 1 hr at 4°C (Invitrogen). Anti-*H. hepaticus* and anti-VgrG were incubated at 20 µg/ml overnight at 4°C. Rabbit anti-mouse E-cadherin antibody was diluted at 1:250 (Santa Cruz Biotech). Samples were imaged using a Zeiss LSM 510 Upright confocal microscope.

Microarray Hybridization and Data Analysis

RNA was prepared using TRIzol. RNA was labeled and hybridized to Agilent microarrays (Whole Mouse Genome Microarray) following the manufacturer's instructions. Microarrays were scanned using an Agilent DNA Microarray Scanner G2565CA, and data were acquired using Agilent's Feature Extraction Software version 10.1.1.1. Significant genes were selected based on $p < 0.05$ and fold change > 1.5 . For enrichment analysis of biological process ontology, probe lists were analyzed in DAVID and selected based on $p < 0.01$.

Animal Housing

Seven- to ten-week-old animals were used for all experiments. SPF C57Bl/6 and C3H/HeJ mice were purchased from Taconic Farms and Jackson Laboratories, respectively. SPF C57Bl/6 *Rag1*^{-/-} and C57Bl/6 *IL-10*^{-/-} mice were bred and maintained in our facilities. Germ-free Swiss Webster and C57Bl/6 mice were kept in sterile isolators. Germ-free animals were screened weekly for bacterial, viral, and fungal contamination. Animals were cared for under established protocols and IACUC guidelines of California Institute of Technology.

IEC Isolation

Colons were cut longitudinally, and 1 cm fragments were incubated twice in HBSS (–Ca²⁺, Mg²⁺) with 5 mM EDTA and 10 mM HEPES for 20 min at 37°C with gentle agitation.

Cells were then treated with 5% FBS, 3 U/ml dispase, and 100 μ g/ml DNase for 30 min at 37°C. IECs were subsequently treated with 200 μ g/ml gentamicin for 2 hr at 37°C, lysed with 0.5% saponin, and plated on TVP plates for selection of *H. hepaticus*.

Adoptive Cell Transfer

Single-cell suspensions of spleens from sex-matched mice were treated with red blood cell lysing buffer (Sigma). CD4⁺ T cells were isolated using a negative selection CD4⁺ isolation kit (Miltenyi Biotec). Cells were stained with 5 μ g/ml anti-CD4-FITC and 2 μ g/ml anti-CD45Rb-PE (eBioscience). CD4⁺CD45Rb^{hi} cells were isolated by fluorescence activated cell sorting (FACS). Cells (2×10^5) were injected intraperitoneally into *Rag1*^{-/-} animals. Two weeks, later mice were orally gavaged with 1×10^8 wild-type, Δ lcmF, or Δ Hcp *H. hepaticus*. Animals were sacrificed 2–4 weeks after. Colon tissues were fixed in Bouin's fixative and sent out for paraffin-embedded sectioning and H&E staining (Pacific Pathology, San Diego).

Quantitative Real-Time PCR

RNA was extracted using TRIzol. RNA was treated with DNase (Sigma) prior to cDNA conversion using iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Reactions were carried out on a Bio-Rad iCycler IQ5. For cytokine analysis, samples were normalized to the housekeeping gene L32.

Colon Organ Culture and MLN Restimulation

Colon tissues were washed in PBS and cultured in 48-well plates in serum-free complete RPMI for 24 hr. Supernatants were collected and normalized to total protein concentration using Bradford reagent. Samples were analyzed by ELISA (eBioscience). For restimulation assays, MLNs were disrupted into single-cell suspensions and cultured in 48-well plates at 1×10^6 cells/ml in complete RPMI. Cell stimulants were added: PMA at 50 ng/ml, ionomycin at 500 ng/ml, anti-CD3 at 2 μ g/ml, and anti-CD28 at 2 μ g/ml. Supernatants were collected after 1 day and analyzed by ELISA.

MODE-K Antigen Presentation

MODE-K cells were treated with 100 U/ml of IFN γ for 7 days prior to experiments. MODE-K cells were pulsed with 20 μ g/ml of SHeAg for 24 hr. Cells were rinsed prior to the addition of CD4⁺ T cells collected from the MLNs of SPF C3H/HeJ mice colonized with wild-type, Δ lcmF, Δ Hcp, or no *H. hepaticus* for 2 weeks. The ratio of MODE-K to T cells was 1:10. After 72 hr, RNA was collected and cytokine levels assayed by qRT-PCR. Preparation of SHeAg consisted of sonicating wild-type bacteria and centrifuging lysate to remove insoluble material. MODE-K cells were stained with anti-MHC class II antibodies obtained from eBioscience or ATCC (10.2.16) and analyzed by flow cytometry.

Statistical Analysis

Student's t test was used for evaluating statistical significance. $p < 0.05$ was considered significant.

ACCESSION NUMBERS

Microarray data have been deposited in the GEO database with the accession number GSE20434.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table, four figures, Supplemental Experimental Procedures, and four movies and can be found with this article at doi:10.1016/j.chom.2010.03.004.

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